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Laverty, G., Gorman, S. P., & Gilmore, B. F. (2013). Biomolecular mechanisms of staphylococcal biofilm formation. *Future microbiology*, *8*(4), 509-24. https://doi.org/10.2217/fmb.13.7

Published in:
Future microbiology

Document Version:
Peer reviewed version

Biomolecular Pathogenesis of Staphylococcal Biofilm Formation

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Keywords: Quorum sensing, adhesion, biomaterial, ica operon, adhesins

Abstract

The multitude of biomolecular and regulatory factors involved in staphylococcal adhesion and biofilm formation owes much to their ability to colonise surface and become the preferential bacterial phenotype. Judging on total number, biomass and variety of environments colonised, bacteria can be categorised as the most successful life form on earth. This is due to the ability of bacteria and other microorganisms to respond phenotypically via biomolecular processes to the stresses of their surrounding environment. This review focuses on the specific pathways involved in the adhesion of the Gram-positive bacteria *Staphylococcus epidermidis* and *Staphylococcus aureus* with reference to the role of specific cell surface adhesins, the *ica* operon, accumulation associated proteins and quorum sensing systems and their significance in medical device related infection.

Main text

Introduction

Microorganisms have been implicated in a variety of problems within the food, oil, paper and medical industries [1]. The ability of microorganisms to attach to surfaces provides an evolutionary advantage allowing maturation, increased survival and symbiotic relationships to be established within the biofilm environment. Upregulation of specific genes allow and the associated molecular processes enable planktonic free-flowing cells to attach to surfaces, aggregate and form a hydrated extracellular polymeric matrix which is phenotypically advantageous for survival [2]. Gram-positive microorganisms such as *Staphylococcus epidermidis* and *Staphylococcus aureus* are present on the skin of humans as part of their resident microflora [3]. In healthy individual they confer a mutualistic benefit with their host

by preventing colonisation of the skin surface by transient pathogenic microorganisms. However in circumstances were the host's immunity becomes impaired, such as trauma associated with medical device implantation, resident bacteria can become opportunistic attaching to the biomaterial surface and forming resistant biofilms. The purpose of this review is to explore the differences and similarities in the molecular processes involved in Gram-positive biofilm formation, with particular relevance to staphylococci. Understanding these processes may provide a means whereby the biofilm's properties of increased resistance to shear stress, superior utilisation of nutrients, energy and increased antimicrobial resistance may be overcome.

Processes involved in Gram-positive biofilm formation: *Staphylococcus epidermidis* and *Staphylococcus aureus*

Staphylococcus epidermidis is the most prevalent biofilm forming coagulase negative staphylococci [4]. Numerous research has been conducted to characterize the various stages, genes and pathways involved in biofilm formation, the majority of these factors are outlined in Figure 1.

Adhesion in staphylococci

Cell surface hydrophobicity and cell surface adhesins

The primary or nonspecific adhesion of staphylococci is due mainly to the cell and cell surface hydrophobicity [5]. In terms of adherence to smooth, abiotic surfaces, such as those present on many biomaterial surfaces, the galactose and glucosamine rich capsular polysaccharide-adhesin is reported to have an important role [6]. Capsular polysaccharide-adhesin is composed of a high molecular weight (28 kDa)

polymer of *β*-1,6-linked *N*-acetylglucosamine residues with O-linked phosphate, succinate and acetate substituents on the amino groups. These groupings confer further hydrophobic character to the *Staphylococcus* bacterial capsule [7]. Another role of capsular polysaccharide-adhesin in staphylococci is to offer protection against the host's immune response, for example complement-mediated antibody-independent opsonic killing, through the physical formation of the slimy bacterial capsule that acts as a barrier to phagocytosis [8]. The glucose rich extracellular slime associated antigen was discovered by Christensen *et al* [9]. Antigenically different to capsular polysaccharide-adhesin, slime associated antigen is also heat and protease stable. It was observed, through characterisation of capsular polysaccharide-adhesin positive and slime associated antigen positive and negative strains, that capsular polysaccharide-adhesin was responsible for the process of surface attachment whereas slime associated antigen is linked to accumulation and biofilm maturation at the surface. Research has shown slime associated antigen to be chemically identical to polysaccharide intercellular adhesin [10]. Both polysaccharide intercellular adhesin and capsular polysaccharide-adhesin share a *β*-1,6-linked-polyglucosamine backbone, with differences occurring in the primary substituent present on the amino groups. They are both synthesized from the proteins encoded by the *ica* operon [11].

The discovery of a Tn*917* insertion mutant of *Staphylococcus epidermidis* by Heilmann *et al* confirmed the importance of hydrophobicity, particularly in relation to plastics [12]. They observed that this mutant was significantly less hydrophobic than a wild type strain (O-47) and thus was unable to adhere to a polystyrene surface. Another Tn*917* mutant was also lacking in four important cell surface adhesins, required for secondary adhesion, but the genetic restoration of one of these adhesins

(of molecular mass 60 kDa) fully restored adherence capabilities and showed the importance of surface bound adhesins in *Staphylococcus* adhesion. The secondary attachment of *Staphylococcus epidermidis* is improved by the presence of the cell adhesion autolysin E, which binds to plasma proteins such as vitronectin present in the conditioning layer formed on implanted biomaterials [13]. The 60-kDa adhesion analysed by Heilmann *et al* was shown to be a proteolytic fragment of autolysin E [14]. Heilmann *et al* are also responsible for the characterisation of a novel autolysin-adhesin in *Staphylococcus epidermidis* [15]. This surface bound novel autolysin-adhesin was shown to be 35kDa in molecular mass and possess bacteriolytic properties, with saturable dose dependent adhesion to fibronectin, fibrinogen and vitronectin also shown *in vitro*. Biofilm formation in *Staphylococcus epidermidis* is not reliant on autolysin and autolysin-adhesin expression alone and it is still unknown whether autolysin E mediates attachment directly or helps to expose alternative adhesins [16].

There are several surface bound proteins in *Staphylococcus epidermidis* that are responsible for binding specifically to collagen, vitronectin, fibronectin and fibrinogen and other proteins present in the extracellular matrix. Included in these proteins together with autolysin and autolysin-adhesin are; the collagen binding extracellular lipase GehD [17]; the large (1 MDa) fibronectin binding protein Embp [18] and the fibrinogen binding proteins and SdrG [19]. Both fibrinogen binding protein and SdrG are members of the same staphylococcal surface protein gene family, sharing similar dipeptide serine-aspartate repeats, sortase cleavage sites, hydrophobic and cationic domains [20]. The gene encoding for fibrinogen binding protein (*fbe*) has been isolated in the majority of *Staphylococcus epidermidis* strains,

with an incidence of 95% in clinical isolates [21]. Fibrinogen binding protein is the only true microbial surface components recognizing adhesive matrix molecule (MSCRAMM) found in *Staphylococcus epidermidis* and although it is present in *Staphylococcus aureus* it also has similar structural and functional properties to clumping factor (ClfA) found in some strains of *Staphylococcus aureus* [22]. Clumping factor A (ClfA) is a cell wall-associated adhesin that mediates binding to fibrinogen and platelets, and although staphylococci share many adhesive properties and mechanisms it has only been isolated in *Staphylococcus aureus* [23]. Similarly the cell-wall protein clumping factor B (ClfB) of *Staphylococcus aureus* aids adhesion and colonisation to squamous epithelial cells present in nasal passages [24]. MSCRAMMs are more prevalent in *Staphylococcus aureus*, including clumping factors A and B (ClfA and ClfB), collagen binding protein and fibronectin binding factors A and B [25]. Binding to fibrinogen by these isolates varies however, leading to the hypothesis that fibrinogen binding protein and other surface adhesins are expressed to different degrees when comparing multiple isolates. Factors such as protease activity, sortase cleavage of the Leu-Pro-Xaa-Thr-Gly (LPXTG) amino acid sequence motif, insufficient length of Shine-Dalgarno repeat region and capsular formation may determine the extent to which adhesins are exposed [26].

The action of sortase, namely sortase A, in staphylococci and other Gram-positives is of importance in the covalent anchoring of surface adhesins to peptidoglycan in the cell wall allowing them to be readily available for attachment [27]. MSCRAMMs such as fibrinogen binding protein are composed of three distinct regions namely; a hydrophobic portion; a charged tail and most importantly a LPXTG motif, where X represents any amino acid [28]. By cleavage of this motif between the threonine and

glycine residues an acyl-enzyme intermediate is formed within the sortase active site, with nucleophilic attack of the amino groups present in the cell wall crosslinks allowing binding of MSCRAMMs to peptidoglycan in the cell wall [29].

The role of teichoic acids

Cell wall teichoic acids are the highest source of polyanionic charge on the staphylococcal bacterial cell envelope [30]. Research has also shown that increased cationic charge provided by incorporation of D-alanine into teichoic acids, an important component of the staphylococcal extracellular matrix, is a determinant in the successful attachment of staphylococci to biomaterials [31][32][33]. The production of teichoic acids is controlled by the *dlt* gene operon; it is this gene sequence that is responsible for D-alanine incorporation [30]. Gross *et al* showed gene mutants of *dlt*, namely *dltA*, that did not incorporate D-alanine were teichoic acid negative and failed to adhere to glass and polystyrene [33]. They concluded that despite other adherence factors being present, including the *ica* operon and polysaccharide intercellular adhesin production, the electrostatic repulsive forces induced by increased cell negativity of staphylococci lead to prevention of bacterial adhesion to polystyrene and glass. Although these results may show some correlation between cell surface charge and electrostatic forces in biofilm formation, there is no conclusive evidence for the activity of *dltA* staphylococcal mutants in other polymers such as Teflon. Research performed by Vergara-Irigaray and colleagues showed cell wall absent teichoic acid mutants to have similar levels of poly-*N*-acetylglucosamine production; a higher degree of cell aggregation but reduced capacity to form biofilms compared with wild type [34]. Attachment with Biofilm formation itself has been shown to be restored with the addition of magnesium but not calcium ions, showing

that the balance of charge at the surface of Gram-positive bacteria is important in determining adhesion and ultimately biofilm formation, with the cationic charge of magnesium ions acting as a direct replacement for that of D-alanine [31][35]. Mutant *dltA* staphylococci have also been shown to be more sensitive to vancomycin and host defence peptides [36].

Accumulation and the *ica* operon in staphylococci

The accumulation of cellular aggregates at the surface of the biomaterial is a key stage in the adhesion of biofilm forming microorganisms in medical device related infection. Approximately 85% of *Staphylococcus epidermidis* strains from infective blood cultures have been shown to possess the *ica* gene cluster [37]. Polysaccharide intercellular adhesin is localized to the cell surface and is the key component for the intercellular adhesion of *Staphylococcus epidermidis*. Together with capsular polysaccharide-adhesin, polysaccharide intercellular adhesin is a product of the *ica* gene operon, the most understood biofilm mediating pathway in staphylococci [38]. Sharing the same linear β-1,6-linked-polyglucosamine backbone as capsular polysaccharide-adhesin, polysaccharide intercellular adhesin can exist as one of two polysaccharides termed polysaccharide intercellular adhesin I or polysaccharide intercellular adhesin II with an average chain length of 130 residues [39]. Deacylated *N*-acetylglucosamine accounts for 15-20% of polysaccharide intercellular adhesin and is essential for its functional properties including the ability to colonize, form biofilms and resist phagocytosis by neutrophils and antibacterial peptides [40]. The *ica* gene operon codes for the proteins and enzymes responsible for polysaccharide intercellular adhesin production.

This *ica* gene cluster can be further differentiated to the *ica*A, *ica*D, *ica*B and *ica*C loci each responsible for relevant pathogenic and virulent factors involved in polysaccharide intercellular adhesin synthesis [40][38][41]. The transcription of the *ica*ADBC gene operon is negatively regulated by an adjacent five nucleotide base *icaR* gene sequence, that itself codes for a transcriptional regulator that binds to the *ica*ADBC promoter [42][35]. Evidence for the role of *icaR* has been verified through deletion of the *icaR* gene corresponding to increased polysaccharide intercellular adhesin production [43]. The proteins transcribed, icaA, icaD, icaB and icaC have separate but correlating functions in polysaccharide intercellular adhesin synthesis (Figure 2). IcaA is a transmembrane protein similar to *N*-acetyl-glucosaminyltransferases and works in tandem with icaD, also positioned on the cytoplasmic membrane, to form *N*-acetyl-glucosamine oligomers with UDP-*N*-acetylglucosamine as a substrate [35]. When both proteins are transcribed oligomers may form to a maximum of 20 residues in length. The presence of the integral membrane protein icaC increases both the length of *N*-acetyl-glucosamine oligomers and allows for the translocation of the polysaccharide through the cytoplasmic membrane to the cell surface [41]. The expression of *icaA*, *icaD* and *icaC* are a necessary requirement for the production of polysaccharide intercellular adhesin, with the deacetylase-like icaB conferring significant functional virulence and cationic charge by deacetylation of the poly-*N*-acetylglucosamine sequence [41][40]. It is likely that an uncharged fully acetylated *N*-acetylglucosamine primary product is produced, with a second icaB protein mediated deacetylation step leading to positively charged *N*-glucosamine oligomers.

This hypothesis has developed from the observation that in *in vitro* synthesis pathways no virulence dependent deacetylated residues have been isolated. There has been much debate as to the location of icaB as many papers hypothesize it to be secreted into the surrounding medium acting as a peptide signal molecule [38][35]. More recently Vuong *et al* obtained results to indicate that icaB interacts with the staphylococcal cell surface through non-covalent means, with its location likely to be in the cell surface matrix [40]. The role of the *ica* gene operon in regulating biofilm formation, adhesion and virulence has been proven by the introduction of the *icaRADBC* sequence into strains of *Staphylococcus epidermidis* that were previously *icaADBC* negative and biofilm negative [13]. The presence of the *icaRADBC* gene cluster allows the production of polysaccharide intercellular adhesin leading to increased biofilm formation when sufficient IcaB protein allows for deacetylation[44][40].

Regulation of *icaR* transcription in *Staphylococcus epidermidis* is controlled by the alternative sigma factor σ^B which itself is positively regulated by the protein RsbU via activation of environmental stresses for example heat, acid, salt or ethanol shock [45]. Also included in this regulatory cascade are; the anti-sigma factor RsbW, the anti-anti sigma factor RsbV, with RsbU acting as a RsbV-specific phosphatase as outlined in Figure 3. This mechanism is true for *Staphylococcus epidermidis* but not *Staphylococcus aureus* [46]. The production of an uncharacterized intermediate protein molecule, σ^B indirectly represses the transcription of the *icaR* operon and its expression is especially important in the stability of *Staphylococcus epidermidis* biofilm under environmental stresses, such as lack of nutrients [47]. Knobloch *et al* proved alterations in the gene responsible for RsbU transcription (*RsbU*), via the use

of a Tn917 insertion mutant, results in a *Staphylococcus epidermidis* strain that cannot express σ^B. It was observed in this class III mutant, labelled M15, that the *icaADBC* operon was not transcribed suggesting σ^B expression is essential for *icaADBC* activity in *Staphylococcus epidermidis* [45]. Both ethanol and high osmolarity (both environmental stresses) have been shown to be inducers of σ^B. Knobloch *et al* also observed that the presence of ethanol could result in the restoration of biofilm formation in mutant M15 but the presence of sodium chloride (NaCl) salt would not restore biofilm formation. However it has also been proposed by Conlon *et al* that *icaADBC* operon activation by ethanol is only *icaR* dependent whereas for NaCl to activate *icaADBC* expression both *icaR* and σ^B activity are required [48]. With these theories in mind two regulatory pathways could exist in *Staphylococcus epidermidis* to control biofilm formation with the ethanol mediated pathway acting independently of σ^B [49]. This alternative ethanol induced pathway could involve activation of σ^B by RsbU substitutes or the formation of polysaccharide intercellular adhesin by a completely different pathway independent of σ^B, as Conlon *et al* suggest [48]. This mechanism may follow that of other biofilm forming staphylococcal species [50]. It is still unclear how responsible σ^B is for the control of *icaADBC* operon transcription as no identifiable σ^B binding site has been identified close to *icaADBC* [51]. One explanation of σ^B control of the *icaADBC* is through the presence of genes that code for staphylococcal accessory regulator, a global regulator that is commonly associated with *Staphylococcus aureus* biofilm development, where σ^B is only essential in a minority of strains [52][53][46].

SarA is an essential element in the synthesis of polysaccharide intercellular adhesin and biofilm development in *Staphylococcus aureus* through the *icaADBC* operon with

environmental signals such as ethanol, salt stress and iron limitation important [54]. For *Staphylococcus aureus* in particular, the staphylococcal accessory regulator protein A has been shown to be positively regulated by σ^B [51]. Although further research by Valle *et al* has shown σ^B negative *Staphylococcus aureus* to still have biofilm forming potential suggesting the production of staphylococcal accessory regulator has still to be characterized fully [46]. 84% of the staphylococcal accessory regulator protein present in *Staphylococcus epidermidis* corresponds to that of *Staphylococcus aureus*, however the regulation of staphylococcal accessory regulator varies due to the differing organisation of staphylococcal accessory regulator promoters at a nucleotide level [55]. Staphylococcal accessory regulator binds to and positively regulates the *icaADBC* operon with high affinity through an *icaR* independent mechanism [56]. The staphylococcal accessory regulator gene has been implicated in the *agr* quorum sensing system of staphylococci but mediates biofilm formation via an *agr* independent pathway [57]. Purine biosynthesis is also associated with *ica* expression and biofilm formation in Gram-positive microorganisms and although no direct binding site for purines or preceding genes that code for purines exist on the *icaADBC* operon, purines may play an indirect role in *icaADBC* regulation [58].

The accumulation associated proteins in staphylococci

The importance of biofilm formation for the survival of *Staphylococcus epidermidis* and staphylococci generally means that the *ica* operon itself is not a necessity for biofilm formation. A number of *ica* independent mechanisms exist as shown by strains of *Staphylococcus epidermidis* lacking *icaADBC* but still forming biofilms [37][59][60]. Accumulation associated protein has been shown to be involved in the

accumulation of *Staphylococcus epidermidis* independently of polysaccharide intercellular adhesin. Past research had deemed accumulation associated protein to be a cell wall receptor for polysaccharide intercellular adhesin [61]. In *Staphylococcus aureus* the surface protein G is homologous to the accumulation associated protein of *Staphylococcus epidermidis*, however although it has been linked to intranasal adhesion of *Staphylococcus aureus* its *in vivo* activity is less characterized than accumulation associated protein [62]. Rohde *et al* proved that limited proteolysis of accumulation associated protein by endogenous serine and metalloproteases and exogenous trypsin, elastase and cathepsin G induced biofilm formation [63]. Proteolytic processing of accumulation associated protein leads to the removal of the N-terminal domain resulting in the exposure of *N*-acetylglucosamine binding domains, also termed G5 domains due to the prominence of glycine residues [64]. Protease production itself is controlled via quorum sensing pathways such as the *agr* and *sarA* in staphylococci, thus biofilm formation via accumulation associated protein is linked to virulence [65].

Quorum sensing in staphylococci:

I. The accessory gene regulator system (*agr*)

Symbiosis, antibiotic production, biofilm formation and virulence are defined by two quorum sensing systems in staphylococci. These are the accessory gene regulator system (*agr*) and the *luxS* system [66][67][68]. The accessory gene regulator system (*agr*) consists of two units RNA-II and RNA-III whose transcription is dependent on the activation of their respective P2 and P3 *agr* promoters [69]. RNA-II consists of four genes *agrB*, *agrD*, *agrC* and *agrA* [70]. The autoinducing peptide backbone is synthesized via transcription of the *agrD* gene. The product of *agrB* transcription is a

protease that cleavages portions of the agrD product to form a thiolactone ring structure (lactone ring in one case) of approximately 8 amino acids in length, otherwise known as autoinducing peptide [71]. AgrC is the sensory kinase of the *agr* quorum sensing system with the binding of a threshold concentration of autoinducing peptide to this transmembrane protein resulting in activation of AgrA via phosphorylation or dephosphorylation (Figure 4). This autoinductive pathway results in RNA-II and RNA-III (the effector molecule of the *agr* system) transcription via the activation of the promoters P2 and P3 by activated AgrA aided by SarA [58].

The activation of the *agr* system correlates to the mid to end point of exponential growth and entry into the stationary phase of growth with the down regulation of cell surface protein related genes but an upregulation in virulence factors [72]. This leads to the production of the regulatory RNA-III molecule that initiates the transcription of genes coding for a variety of virulent proteins (toxins) including enterotoxin B also known as *Staphylococcus aureus* exoprotein expression regulator and *Staphylococcus* serine proteases and *Staphylococcus* proteases (spr) and controls the downregulation of genes encoding cell surface proteins and adhesion, for example *Staphylococcus* protein A and fibronectin-binding [73][74]. The overall picture is not as simplistic however, as research conducted by Vuong *et al* has shown the genes coding the adhesin autolysin E (*altE*) are upregulated by *agr* quorum sensing pathways in *Staphylococcus epidermidis* and *sarA* appears upregulated similarly in *Staphylococcus aureu*s thereby increasing biofilm formation [72]. However as stated previously staphylococcal accessory regulator gene has been implicated in the *agr* quorum sensing system of staphylococci but mediates biofilm formation via an *agr* independent pathway [57]. The possibility still remains that *agr* may mediate

adhesion in *Staphylococcus epidermidis* strains particularly in reference to biomaterials [13]. As intercellular adhesion in staphylococci is influenced by polysaccharide intercellular adhesin production, it has been shown that the *luxS* quorum sensing system, not *agr*, has a role in down-regulating this process [67].

The importance of *agr* to the biofilm process is greatest at the detachment phase of growth [75][76]. Wild type staphylococci that utilize *agr* have biofilms that are less thick than *agr* negative mutants due to an ability to detach from the matured biofilm, rather than decreased microbial growth [77]. Detachment in both *Staphylococcus epidermidis* and *Staphylococcus aureus* occurs due to the production of short amphipathic peptides known as phenol-soluble modulins, such as δ-toxin, encoded by regulatory RNA-III molecule and mediated by the *agr* regulatory system. These peptides themselves have no autoinducing or regulatory affect on the *agr* system [76].

The ability of microorganisms to coordinate a range of actions and phenotypic traits, via a process such as quorum sensing, shows that this mechanisms itself may be a specific target in reducing biofilm formation and virulence associated with medical device related pathogens [78][79]. Research by Balaban *et al* have shown that RNA-III inhibiting peptide has significant activity in preventing *Staphylococcus epidermidis* and *Staphylococcus aureus* biofilm formation using an *in vivo* rat Dacron graft model [80]. RNA-III inhibiting peptide targets RNA-III activating protein, to prevent the phosphorylation of the protein target of RNA-III activating protein. The release of RNA-III activating protein and phosphorylation of the protein target of RNA-III activating protein is itself a quorum sensing process leading to the formation of numerous surface adhesion proteins, together with the autoinducing expression of

the *agr* operon controlling biofilm formation in staphylococci (Figures 5 and 6) [81]. RNA-III inhibiting peptide itself is a heptapeptide of structure of amide form, YSPWTYNF-NH_2, is non-pathogenic as it inhibits cell to cell communication via competing for binding sites on the protein target of RNA-III activating protein but it is not bactericidal [82].

II. Quorum sensing in staphylococci: the *luxS* system

Whereas the *agr* system has no effect on the *icaADBC* gene operon and polysaccharide intercellular adhesin formation the presence of an alternative quorum sensing *luxS* has been linked to preventing the production of polysaccharide intercellular adhesin in staphylococci via downregulation of *icaADBC* [83]. Present in both Gram-positive and Gram-negative bacteria the *luxS* quorum sensing system results in the formation of autoinducing peptide-II [84][85][86]. *LuxS* and *agr* absent mutants both shared the common properties of forming thicker but less virulent biofilms than wild type strains of *Staphylococcus epidermidis* [67]. This research by Xu *et al* claimed that thinner biofilm growth in *luxS* positive strains was due to a downregulation in the *icaADBC* operon rather than cellular metabolic processes as there were no noticeable differences in the growth patterns of *luxS* negative and positive strains. This contrast to the theory put forward by Vendeville *et al* who observed that *luxS* is involved in the activated methyl cycle and thus may alter the metabolism and biofilm formation of bacteria [87].

The synthesis of autoinducer-II occurs in three enzyme enzymatic steps. The substrate molecule is *S*-adenosylmethionine, a molecule found as a cofactor for many DNA- and RNA-linked processes including protein synthesis. The presence of

methyltransferases results in *S*-adenosylmethionine donating methyl groups to a variety of substrates in the methyl cycle to form the toxic intermediate *S*-adenosylhomocysteine. The nucleosidase enzyme Pfs (5′methylthioadenosine/*S*-adenosylhomocysteine nucleosidase) mediates the hydrolysis of *S*-adenosylhomocysteine to *S*-ribosylhomocysteine via the loss of adenine. At this stage the transcription of *luxS* with the formation of LuxS leads to the catalysis of *S*-ribosylhomocysteine cleavage to 4,5-dihydroxy 2,3-pentanedione and homocysteine [88]. The production of 4,5-dihydroxy 2,3-pentanedione to autoinducing peptide-II is relatively uncharacterized in the literature with Xavier *et al* stating that 5-dihydroxy 2,3-pentanedione cyclizes to form pro-autoinducer-II, and subsequently boron is added to form autoinducer-II in Gram-negative *Vibrio harveyi*. A similar mechanism may exist in Gram-positives also (Figure 7) [85].

Conclusions

Biofilms are particularly prevalent in marine ecosystems where they constitute more than 99.9% of bacteria present with these results correlating to the majority of ecosystems [89]. This suggests a selective evolutionary advantage for biofilm forming microorganisms over planktonic forms [25]. Infections of medical devices are a significant problem due to their high impact on patient morbidity, mortality and monetary expenditure. Most device related infections are due to contamination of the device from environmental pathogens, such as staphylococcal skin flora, both before and during implantation [90]. Biomolecular processes form a viable target by which treatment strategies may be developed to prevent bacterial adherence and transfer from planktonic to more resistant biofilm forms. In Gram-positive bacteria potential treatment strategies include influencing the *agr* and *luxS* quorum sensing systems.

Inhibiting the *agr* quorum sensing signal has been show to increase attachment and biofilm production in both *Staphylococcus epidermidis* and *Staphylococcus aureus* [72][91]. This contrasts to what is seen with quorum sensing systems in Gram negative microorganisms such as *Pseudomonas aeruginosa* [92], further evidence that increased study is required in this area to positively affect clinical outcomes. For staphylococcal biofilms, future work will be required to focus on the specific role and action of teichoic acids, present at high density throughout the biofilm matrix, cell surface adhesins and MSCRAMMs as promising drug targets for vaccine development. For example Stranger-Jones *et* al, showed a vaccine containing the MSCRAMMs IsdA, IsdB, SdrD, and SdrE were identified as protective in a murine model of *Staphylococcus aureus* abscess formation [93]. Inhibition of sortase A has been hypothesised as a possible target for the prevention of surface protein anchoring to the peptidoglycan cell wall and adhesin exposure with several distinct sortase inhibitor classes identified whose aims are to irreversibly modify the thiol active site of sortase [94][95].

Future Perspectives

The need to prevent bacterial adherence and eradicate existing established biofilms is an increasing challenge for an innovative scientific community whose antimicrobial arsenal is updating at a diminishing rate. Over the coming years the study of bacterial biomolecular processes may hold the key to producing effective future antimicrobial strategies that are targeted specifically to eradicate pathogenic bacteria thus allowing mutualistic commensal bacteria to thrive in the host environment. Such an approach would resolve infection, meet treatment goals and reduce potential systemic side effects, all without the threat of increased antimicrobial resistance. In order to

achieve these goals bacterial genotypes must be systematically linked to both resistance and biomolecular pathways thereby allowing optimum processes to be targeted. In order to be of greater success clinically and to reduce the potential for resistance to develop, such biomolecular strategies will likely be required to be utilised concurrently with novel biocidal approaches such as the use of antimicrobial peptides [96] or ionic liquids [97].

Executive Summary

Introduction

The ability of bacteria such as *Staphylococcus epidermidis* and *Staphylococcus aureus* to produce exopolysaccharide biofilms allows for increased survival, maturation and symbiotic relationships to be established at a solid surface environment such as that present on a medical device.

Processes involved in Gram-positive biofilm formation: *Staphylococcus epidermidis* and *Staphylococcus aureus*

The biomolecular processes involved in formation of staphylococcal biofilms can be divided into 5 key areas:

1) **Adhesion in staphylococci: Cell surface hydrophobicity and cell surface adhesins**
    - The primary or nonspecific adhesion of staphylococci is due mainly to the cell and cell surface hydrophobicity.
    - Capsular polysaccharide-adhesin is responsible for the process of surface attachment. Slime associated antigen is linked to accumulation and biofilm maturation at the surface.

- They are both synthesized from the proteins encoded by the *ica* operon.
- The secondary attachment of *Staphylococcus epidermidis* is improved by the presence of the cell adhesin autolysin E, which binds to plasma proteins such as vitronectin present in the conditioning layer formed on implanted biomaterials.
- There are several surface bound proteins in *Staphylococcus epidermidis* that are responsible for binding specifically to collagen, vitronectin, fibronectin and fibrinogen and other proteins present in the extracellular matrix.

2) The role of teichoic acids

- Cell wall teichoic acids are the highest source of polyanionic charge on the staphylococcal bacterial cell envelope.
- Increased cationic charge is provided by incorporation of D-alanine into teichoic acids. This is a determinant in the successful attachment of staphylococci to biomaterials.
- The production of teichoic acids is controlled by the *dlt* gene operon

3) Accumulation and the *ica* operon in staphylococci

- The *ica* gene operon codes for the proteins and enzymes responsible for polysaccharide intercellular adhesin production.
- The *ica* gene cluster can be differentiated into the *ica*A, *ica*D, *ica*B and *ica*C loci each responsible for relevant pathogenic and virulent factors involved in polysaccharide intercellular adhesin synthesis.
- The role of the *ica* gene operon in regulating biofilm formation, adhesion and virulence has been proven by the introduction of the *icaRADBC* sequence into

strains of *Staphylococcus epidermidis* that were previously *icaADBC* negative and biofilm negative.

- Regulation of *icaR* transcription in *Staphylococcus epidermidis* is controlled by the alternative sigma factor σ^B which itself is positively regulated by the protein RsbU via activation of environmental stresses.
- SarA is an essential element in the synthesis of polysaccharide intercellular adhesin and biofilm development in *Staphylococcus aureus* through the *icaADBC* operon it is influenced by environmental signals such as ethanol, salt stress and iron limitation.
- The staphylococcal accessory regulator protein A has been shown to be positively regulated by σ^B.

4) The accumulation associated proteins in staphylococci

- The *ica* operon itself is not a necessity for biofilm formation.
    - Accumulation associated protein has been shown to be involved in the accumulation of *Staphylococcus epidermidis* independently of polysaccharide intercellular adhesin.
    - Accumulation associated protein is a cell wall receptor for polysaccharide intercellular adhesin.
    - Proteolytic processing of accumulation associated protein leads to the removal of the N-terminal domain by proteases resulting in the exposure of *N*-acetylglucosamine binding domains.
    - Protease production is controlled via quorum sensing pathways such as the *agr* and *sarA* in staphylococci.

5) Quorum sensing in staphylococci:

Two quorum sensing systems exist in staphylococci:

I. The accessory gene regulator system (*agr*)

- The accessory gene regulator system (*agr*) consists of two units RNA-II and RNA-III. Transcription is dependent on the activation of their respective P2 and P3 *agr* promoters.
- RNA-II consists of four genes *agrB, agrD, agrC* and *agrA*.
- An autoinductive pathway results in RNA-II and RNA-III (the effector molecule of the *agr* system) transcription via the activation of the promoters P2 and P3 by activated AgrA aided by SarA.
- Staphylococcal accessory regulator gene has been implicated in the *agr* quorum sensing system of staphylococci but mediates biofilm formation via an *agr* independent pathway.
- The importance of *agr* to the biofilm process is greatest at the detachment phase of growth.
- Detachment in staphylococci occurs due to the production of short amphipathic peptides known as phenol-soluble modulins, e.g. δ-toxin, encoded by regulatory RNA-III molecule and mediated by the *agr* regulatory system.

II. Quorum sensing in staphylococci: the *luxS* system

- *luxS* has been linked to preventing the production of polysaccharide intercellular adhesin in staphylococci via downregulation of *icaADBC*
- The *luxS* quorum sensing system is present in both Gram-positive and Gram-negative bacteria and results in the formation of autoinducing peptide-II

- The synthesis of autoinducer-II occurs in three enzyme enzymatic steps.

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837 **Reference annotations**

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Presenting emerging evidence for the existence of *ica*-independent biofilm mechanisms in both *Staphylococcus aureus* and *Staphylococcus epidermidis*.

Financial disclosure/Acknowledgements

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.